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10/519680

β-Sheet breaking peptides

Field of Invention

The invention relates to the field of β-sheet breaking peptides, particularly their use in the treatment of diseases such as Alzheimer's disease, Dementia pugilistica (including head trauma), Hereditary Cerebral Haemorrhage with amyloidosis of the Dutch type (HCHWA-D) and vascular dementia with amyloid angiopathy.

Background of the Invention

Alzheimer's disease (AD), first described by the Bavarian psychiatrist Alois Alzheimer in 1907, is a progressive neurological disorder that begins with short-term memory loss and is characterized by a progressive decline in cognitive function and behaviour. Progression of the disease leads to disorientation, impairment of judgment, reasoning, attention and speech and, ultimately, dementia. The course of the disease usually leads to death in a severely debilitated, immobile state between four and 12 years after onset. AD has been estimated to afflict 5 to 11 percent of the population over age 65 and as much as 47 percent of the population over age 85. The societal cost for managing AD is very high, primarily due to the extensive custodial care required for AD patients. Despite continuous efforts aimed at understanding the physiopathology of AD, there is currently no treatment that significantly retards the progression of the disease.

Pathologically, AD is characterized by the presence of distinctive lesions in the victim's brain, revealed on autopsy. These brain lesions include abnormal intracellular filaments called neurofibrillary tangles (NTFs) and extracellular deposits of amyloidogenic proteins in senile, or amyloid, plaques. Amyloid deposits are also present in the walls of cerebral blood vessels of AD patients. The major protein constituent of amyloid plaques has been identified as a 4.3 kiloDalton peptide called β-amyloid peptide (Aβ)[1]. Diffuse deposits of Aβ are frequently observed in normal adult brains, whereas AD brain tissue is characterized by more compacted, dense-core β-amyloid plaques.[2] These observations suggest that Aβ deposition precedes, and contributes to, the destruction of neurons that occurs in AD[3]. In further support of a direct pathogenic role for Aβ, β-amyloid has been shown to be toxic to mature neurons, both in culture and *in vivo*[4].

Patients with hereditary cerebral haemorrhage with amyloidosis-Dutch-type (HCHWA-D), which is characterized by diffuse β-amyloid deposits within the cerebral cortex and cerebrovasculature, have been shown to have a point mutation that leads to an amino acid substitution within Aβ.[5]

Aβ has also been implicated in vascular dementia with amyloid angiopathy[6] and dementia pugilistica.[7]

Natural Aβ is derived by proteolysis from a much larger protein called the amyloid precursor protein (APP)[8]. The APP gene maps to chromosome 21, thereby providing an explanation for the β-amyloid deposition seen at an early age in individuals with Down's syndrome, which is caused by trisomy of chromosome 21[9].

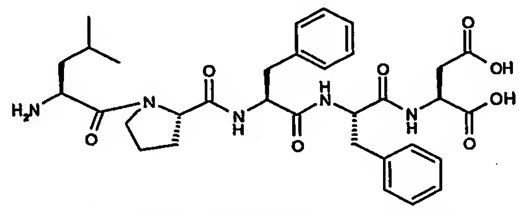
Naturally-occurring Aβ, derived from proteolysis of APP, is 39 to 43 amino acid residues in length, depending on the carboxyl-terminal end point, which exhibits heterogeneity. The predominant circulating form of Aβ in the blood and cerebrospinal fluid of both AD patients and normal adults is Aβ1-40[10]. However, $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}43}$ are also found in β-amyloid plaques[11].

Considerable evidence has accumulated that the pathogenicity of Aβ results from a change in protein conformation[12]. It is believed that a critical event leading to pathology in Alzheimer's disease, Vascular Dementia with amyloid angiopathy and HCHWA-D is the refolding of a natural and non-pathogenic protein, to yield a pathogenic form. The refolding alters the secondary and tertiary structure of the protein without changing its primary structure.

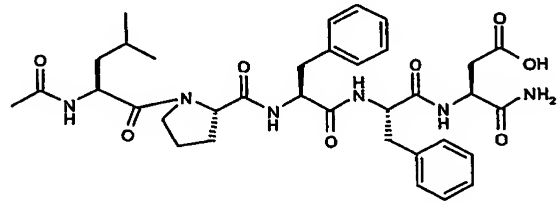
Amyloid is a generic term that is applied to fibrillar aggregates that have a common structural motif: a β-pleated sheet conformation[13]. These aggregates exhibit special tinctorial properties, including the ability to emit a green birefringent glow after staining with Congo red, and the capacity to bind the fluorochrome Thioflavin S[14]. These tinctorial properties form the basis of assays used to detect β-amyloid deposits.

One approach to the treatment and prevention of Alzheimer's disease has been to develop short peptides having some sequence homology to the natural protein sequence believed to

be involved in amyloid formation, but also having one or more amino acids that disfavour or destabilise the formation of β-pleated sheet conformations[15]. The peptides prevent the aggregation of β-amyloid, and thereby prevent its cytotoxic effects. This approach has been suggested in Alzheimer's disease and in prion-related disorders[16,17] and has lead to the β-sheet breaking peptides shown below, amongst others:



WO 96/39834 (New York Univeristy)



WO 01/34631 (Axonyx, Inc.)

US 6,319,498 (Praecis Pharmaceuticals) proposes β-sheet breaking peptides based on Aβ, and exemplifies amino-terminally-biotinylated peptides. US 6,303,567 (Praecis Pharmaceuticals) proposes peptides based on the β-amyloid peptide, but consisting entirely of *D*-amino acids, as β-sheet breaking peptides.

Others have proposed for preventing β-strand association, peptides comprising a β-strand-forming section of peptide which forms a β-strand and associate as such with a target β-strand, and wherein the β-strand forming section of peptide comprises a sequence of at least four consecutive α-L-amino acid, all of which sterically permit the β-stand forming section of peptide to form a β-stand, and at least one of which is a Nα-substituted α-L-amino-acid residue, and any two successive Nα-substituted α-L-amino-acid residues are separated by an odd number of consecutive Nα-substituted α-L-amino-acid residues.[18] Alternatively, peptides containing short β-strands and N-methyl amino acids at alternate positions with or without N-α-acetylated amino-acids have also been developed for inhibition of fibril formation.[19]

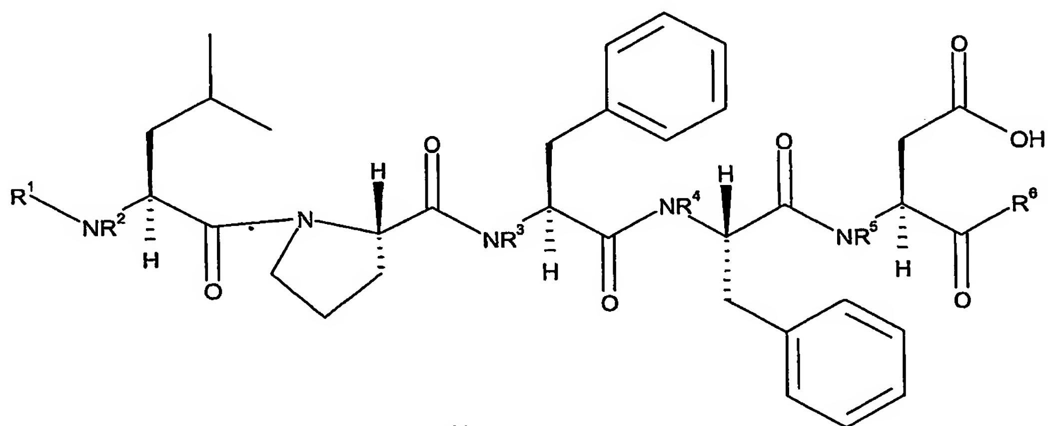
While the known β-sheet breaking peptides have provided valuable information and may have utility in treating Alzheimer's disease, the development of peptide drugs is severely limited by the fact that natural peptides are subject to degradation and rapid metabolism in the gut, the liver and in circulation. Furthermore, the desired site of action for treatment of many amyloid-related disorders is in the brain, and peptides, like many other molecules, may

have difficulty penetrating the blood brain barrier. The brain itself is also replete with peptidases, which degrade peptide molecules.

Summary of the invention

It is an object of the invention to provide a β-sheet breaking peptide with improved pharmacological profile.

In a first aspect, the invention provides a compound of the general Formula I:



(I)

wherein:

R^1 is selected from H and optionally substituted C_2-C_6 -acyl, preferably acetyl;

R^2, R^3, R^4 and R^5 are independently selected from H and optionally substituted C_1-C_6 -alkyl and wherein at least one among R^2, R^3, R^4 and R^5 is optionally substituted C_1-C_6 -alkyl, preferably methyl;

R^6 is selected from OH and NR^7R^8, wherein R^7 and R^8 are independently H and optionally substituted C_1-C_6 -alkyl, preferably NH_2; and salts and tritiated derivatives thereof.

In a second aspect, the invention provides a compound of Formula I for use as a medicament.

In a third aspect, the invention provides a pharmaceutical composition comprising a compound of Formula I, together with a pharmaceutically acceptable excipient or carrier.

In a fourth aspect, the invention provides a use of a compound of Formula I for the preparation of a medicament for the treatment or prevention of a disease or condition selected from Alzheimer's disease, Dementia pugilistica (including head trauma), Hereditary

Cerebral Haemorrhage with amyloidosis of the Dutch type (HCHWA-D) and vascular dementia with amyloid angiopathy.

In a fifth aspect, the invention provides a use of a compound of Formula I for the treatment or prevention of a disease or condition selected from Alzheimer's disease, Dementia pugilistica (including head trauma), Hereditary Cerebral Haemorrhage with amyloidosis of the Dutch type (HCHWA-D) and vascular dementia with amyloid angiopathy.

In a sixth aspect, the invention provides a method of treating Alzheimer's disease, Dementia pugilistica (including head trauma), Hereditary Cerebral Haemorrhage with amyloidosis of the Dutch type (HCHWA-D) and vascular dementia with amyloid angiopathy, comprising administering to a patient in need thereof an effective amount of a compound of Formula I.

In a seventh aspect, the invention provides a use of a compound of Formula I for the preparation of a medicament for the treatment of a disease associated with abnormal protein folding into amyloid and amyloid-like deposits.

In an eighth aspect, the invention provides a use of a compound of Formula I for the treatment of a disease associated with abnormal protein folding into amyloid and amyloid-like deposits.

In a ninth aspect, the invention provides a method of treating a disease associated with abnormal protein folding into amyloid and amyloid-like deposits, comprising administering to a patient in need thereof an effective amount of a compound of Formula I.

Brief description of the drawings

Figure 1 shows the *in vitro* activity of compounds of the invention using a fibrillogenesis assay. The compounds were screened for activity using an assay based on quantitating amyloid fibril formation of synthetic $A\beta_{1-42}$. $A\beta_{1-42}$ (110 μM) in 0.1 M Tris, pH 7.4 was incubated alone or in the presence of a ten-fold molar excess of each of the compounds during 5 days at 37°C. Thereafter, the amount of amyloid fibrils was measured using the Thioflavin T fluorescent method, as described in the Examples. Values correspond to the

percentage of inhibition of amyloid formation and are expressed relative to the activity of the comparative compound of Example 8 (100%).

Figure 2 shows a graph representing the calculated % of injected dose of compound of Example 7 taken up per g of brain vs. time. Mice are injected with 0.2 ml of lactate Ringer's solution containing 1% BSA and tritium labelled peptide ("hot") of Example 7 (100 000 cpm/ml of tritium radioactive peptide).

Figure 3A shows the *in vivo* activity of the compound of Example 1 and the comparative compound of Example 8 in a rat model of cerebral amyloidosis. A representative image shows the difference in amyloid size between the groups of animals treated with vehicle, the comparative compound of Example 8 and the compound of Example 1.

Figure 3B shows a graph depicting the amyloid area in the different animals as estimated by image analysis after immunohistochemistry.

Detailed description of the invention

The compounds of the invention are β-sheet breaking peptides with improved pharmacological profile over known β-sheet breaking peptides.

β-sheet breaking activity can be detected using, for example, an *in vitro* assay, such as that described by Soto *et al.*[14], which measures the ability of test compounds to prevent amyloid fibril formation.

Amyloid fibrils are cytotoxic, inducing cell death by apoptosis[20]. Compounds of the invention can be tested for their ability to prevent cell death induced by amyloid fibrils. Results are reported in the Examples.

A compound having an improved pharmacological profile is considered to be a compound having an increased *in vitro* activity, as measured by either or both of the *in vitro* assays described herein, an increased stability in plasma and/or in brain homogenate, or an increased ability to prevent amyloid deposition *in vivo* in rat brain, as compared with known

compounds. "Improved" encompasses those compounds showing an increase in any one of these parameters, or in more than one. Preferably the improvement will be a statistically significant one, preferably with a probability value of < 0.05. Methods of determining the statistical significance of results are well known and documents in the art, and any appropriate method may be used.

In a preferred group of compounds of Formula I, R^1 is selected from formyl, acetyl, propionoyl and butyroyl. Particularly preferably R^1 is acetyl.

In another preferred group of compounds of Formula I, R^6 is NHMe or NH_2, particularly preferably NH_2.

A particular embodiment of the invention includes compounds of Formula I wherein R^2, R^3, R^4 and R^5 are selected from H, methyl and ethyl, particularly preferably H and methyl and at least one among R^2, R^3, R^4 and R^5 is selected from methyl and ethyl, preferably methyl.

In a particularly preferred group of compounds of Formula I, R^3 is methyl, R^2 is H and R^4 and R^5 are selected from H, methyl.

In another particularly preferred group of compounds, R^3 is methyl and R^2, R^4 and R^5 are H.

In another particularly preferred embodiment, the stereochemistry at all the α-carbon atoms is L;

The compounds of the invention may be isolated and purified as salts. Such salts fall within the scope of the invention. For the purposes of administration to a patient, it is desirable that the salts be pharmaceutically acceptable.

"C_1-C_6 -alkyl" refers to monovalent branched or unbranched alkyl groups having 1 to 6 carbon atoms. This term is exemplified by groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, tert-butyl, pentyl, hexyl and the like.

"C_1-C_5-alkyl" refers to monovalent branched or unbranched alkyl groups having 1 to 5 carbon atoms. This term is exemplified by groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, tert-butyl, pentyl and the like.

"C_2-C_6 Acyl" refers to a group –C(O)R where R includes "C_1-C_5-alkyl" groups. This term is exemplified by groups such as formyl, acetyl, propionoyl and butyroyl.

"Pharmaceutically acceptable salts" refers to salts of the compounds of Formula I that retain the desired biological activity. Examples of such salts include, but are not restricted to, acid addition salts formed with inorganic acids (e.g. hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like), and salts formed with organic acids such as acetic acid, oxalic acid, tartaric acid, succinic acid, malic acid, fumaric acid, maleic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalene sulfonic acid, naphthalene disulfonic acid, and polygalacturonic acid. Said compounds can also be administered as pharmaceutically acceptable quaternary salts known by a person skilled in the art, which specifically include the quaternary ammonium salts of the formula –NR, R', R" $^+$ Z^-, wherein R, R', R" is independently hydrogen, alkyl, or benzyl, and Z is a counter ion, including chloride, bromide, iodide, alkoxide, toluenesulfonate, methylsulfonate, sulfonate, phosphate, or carboxylate (such as benzoate, succinate, acetate, glycolate, maleate, malate, fumarate, citrate, tartrate, ascorbate, cinnamate, mandelate, and diphenylacetate).

When employed as pharmaceuticals, the compounds of the invention are typically administered in the form of a pharmaceutical composition. Such compositions can be prepared in a manner well known in the pharmaceutical art and comprise at least one active compound. Generally, the compounds of the invention are administered in a pharmaceutically effective amount. The amount of the compound actually administered will typically be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the like.

The pharmaceutical compositions of the invention can be administered by a variety of routes including oral, rectal, transdermal, intrathecal, subcutaneous, intravenous, intramuscular, and intranasal. Preferably the compounds of the invention are administered by subcutaneous, intramuscular or intravenous injection or infusion.

In a preferred embodiment of the invention, a compound of the invention is fused to a carrier molecule, a peptide or a protein that promotes the crossing of the blood brain barrier ("BBB"). This serves for proper targeting of the molecule to the site of action in those cases, in which the CNS is involved in the disease. Modalities for drug delivery through the BBB entail disruption of the BBB, either by osmotic means or biochemically by the use of vasoactive substances such as bradykinin. Other strategies to go through the BBB may entail the use of passive diffusion and the use of endogenous transport systems, including carrier-mediated transporters such as glucose and amino acid carriers; receptor-mediated transcytosis for insulin or transferrin; adsorptive-mediated transcytosis. Strategies for drug delivery behind the BBB further include intracerebral implantation.

Depending on the intended route of delivery, the compounds may be formulated as injectable or oral compositions. The compositions for oral administration can take the form of bulk liquid solutions or suspensions, or bulk powders. More commonly, however, the compositions are presented in unit dosage forms to facilitate accurate dosing. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a pre-determined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient. Typical unit dosage forms include pre-filled, pre-measured ampoules or syringes of the liquid compositions or pills, tablets, capsules or the like in the case of solid compositions. In such compositions, the compound of the invention is usually a minor component (from about 0.1 to about 50% by weight or preferably from about 1 to about 40% by weight) with the remainder being various vehicles or carriers and processing aids helpful for forming the desired dosing form.

Liquid forms suitable for oral administration may include a suitable aqueous or non-aqueous vehicle with buffers, suspending and dispensing agents, colorants, flavours and the like. Solid forms may include, for example, any of the following ingredients, or compounds of a

similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatine; an excipient such as starch or lactose; a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavouring agent such as peppermint, methyl salicylate, or orange flavouring.

Injectable compositions are typically based upon injectable sterile saline or phosphate-buffered saline or other injectable carriers known in the art.
The above-described components for orally administered or injectable compositions are merely representative. Further materials as well as processing techniques and the like are known to the skilled practitioner.[21]
The compounds of this invention can also be administered in sustained release forms or from sustained release drug delivery systems. A description of representative sustained release materials is also known to the skilled practitioner.[22,23,24]

The compounds of the invention prevent the aggregation of Aβ associated with the onset and progression of Alzheimer's disease, Dementia pugilistica (including head trauma), Hereditary Cerebral Haemorrhage with amyloidosis of the Dutch type (HCHWA-D) and vascular dementia with amyloid angiopathy. In a preferred method of use of the compounds, administration of the compounds is by injection or infusion, at periodic intervals. The administration of a compound of the invention should preferably begin before any symptoms are detected in the patient, and should continue thereafter. Patients at a high risk for developing Alzheimer's disease, Hereditary Cerebral Haemorrhage with amyloidosis of the Dutch type (HCHWA-D) and vascular dementia with amyloid angiopathy include those with a familial history of these diseases.
Still a further aspect of the present invention is a process for preparing the compounds of Formula I. The compounds of the invention may be prepared from readily available starting materials using the following general methods and procedures. It will be appreciated that where typical or preferred experimental conditions (i.e., reaction temperatures, time, moles of reagents, solvents, etc.) are given, other experimental conditions can also be used unless otherwise stated. Optimum reaction conditions may vary with the particular reactants or solvent used, but such conditions can be determined by one skilled in the art by routine optimisation procedures.

The compounds of the invention may be prepared using methods of peptide synthesis known to the skilled practitioner.[25] In a preferred embodiment, the compounds of the invention are synthesised using solid-phase methods.

A preferred route to the compounds of the invention is depicted in Scheme 1, and particular examples are given in the Examples that follow.

Abbreviations:

The following abbreviations are hereinafter used in the accompanying examples:

min (minute), hr (hour), g (gram), mg (milligram), mmol (millimole), eq (equivalent), ml (milliliter), µl (microliter), mm (millimeter), nm (nanometer), µm (micrometer), Å (Angström), cpm (counts per minute), Ci (Curies), i. v. (intra veinous), ACN (acetonitrile), BBB (Blood Brain Barrier), BOP (benzotriazol-1-yl-oxcy-tris-(dimethylamino)-phosphonium hexafluorophosphate), BOP-Cl (bis(2-oxo-3-oxazoldinyl) phosphinic chloride), Boc (butoxycarbonyl), BSA (Bovine Serum Albumin), Cbz (carboxybenzyl), DCM (dichloromethane), DIC (diisopropyl carbodiimide), DCC (dicyclohexylcarbodiimide), DIEA (diisopropyl ethylamine), DMAP (4-dimethylamino-pyridine), DMF (dimethylformamide), DMSO (Dimethyl Sulfoxide), EDC (1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydro-chloride), EtOAc (ethyl acetate), Et2O (diethylether), Fmoc (9-fluorenylmethoxycarbonyl), HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), HOBt (1-hydroxybenzotriazole), rt (room temperature), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), PBS (Phosphate Buffered Saline), PyBOP (benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluoro-phosphate), PyBrop (bromo-tris-pyrrolidino-phosphonium hexafluorophosphate), TBTU (*O*-benzotriazolyl-*N*,*N*,*N*',*N*'-tetramethyl-uronium-tetrafluoro-borate), TEA (triethylamine), TFA (trifluoro-acetic acid), THF (tetrahydrofuran), ThT (Thioflavine T).

N(Me), in the peptide sequence stands for a methyl group branched on the N atom of the amino acid.

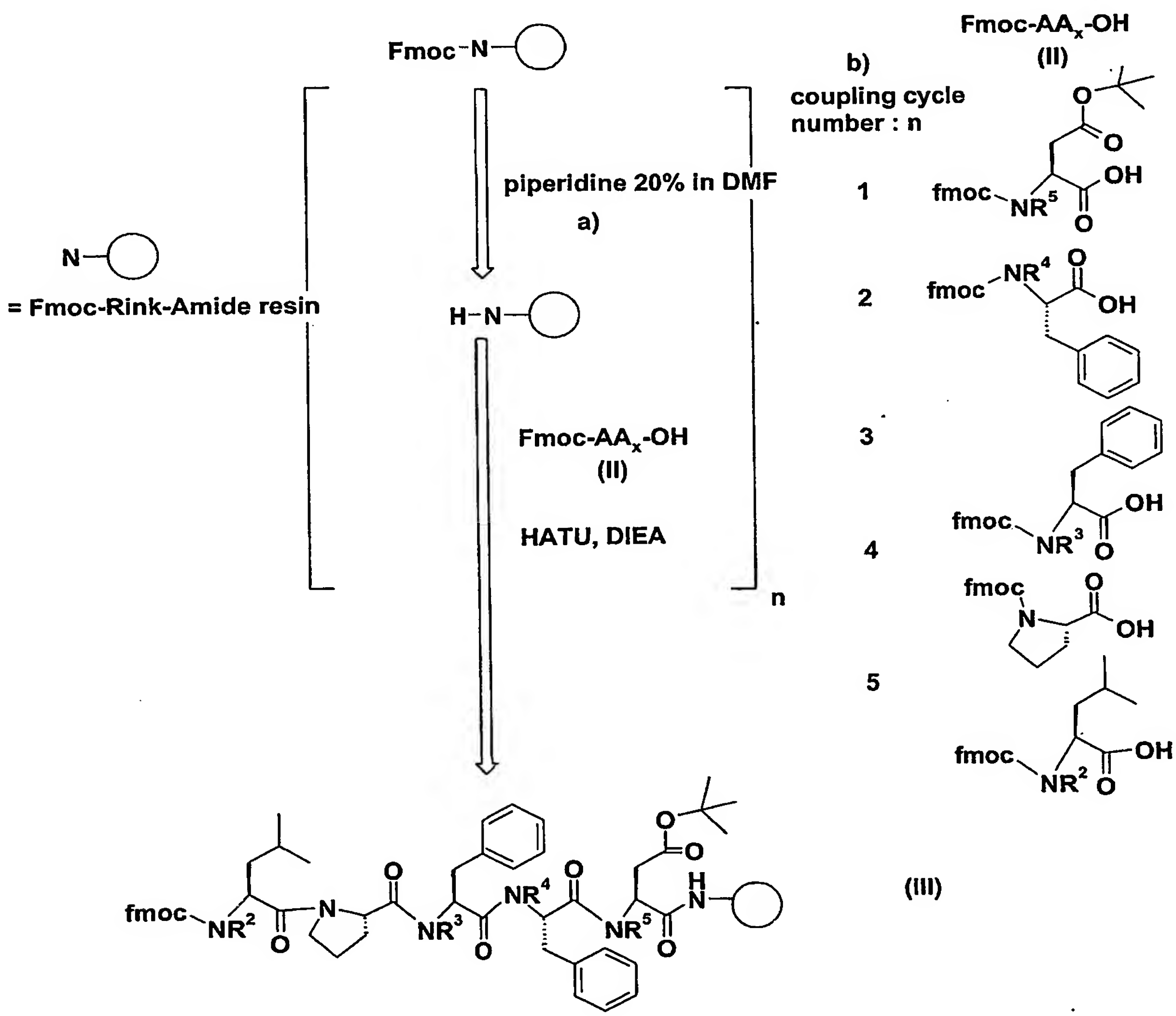
Synthesis of compounds of the invention:

The compounds according to formula I may be prepared from readily available starting materials. Examples of synthetic pathways will be described below.

General protocol :

A preferred pathway for preparing pentapeptides according to the general Formula I, wherein R^2, R^3, R^4 and R^5 are defined above is described in Scheme 1.

Scheme 1:



In a preferred embodiment the peptides of Formula (I) may be synthesized on a solid support using, for example, preferred Rink-Amide resin.

Typically the resin is washed with DCM, DMF, THF and again DCM three times.

a) Resin deprotection step:

A solution of piperidine in DMF (20-50%) is applied to remove the Fmoc-protecting group from the resin. The resin is gently shacked for 30 minutes to 1hour in such a solution. After draining, the resin is washed with DCM, DMF, THF and again DCM each three times.

b) Coupling step:

A solution of Fmoc-aspartic acid (β-O-t-butyl) and the resin are shaken with 1.5 equivalents of coupling reagent, such as PyBOP, TBTU, HATU, BOP-Cl, PyBrop, BOP, EDC, DIC, DCC, preferably HATU, and 3 equivalents of a tertiary base, such as NMP, triethylamine, diisopropyl-ethylamine or any tertiary base with similar pKa, preferably diisopropylethylamine. After 2 to 15 hours at between 25 to 40°C, the resin is drained and washed with DCM, DMF, THF and again DCM three times.

For the remaining four amino acids the deprotection and coupling steps (Scheme I, coupling cycles 2 to 5) are applied sequentially in the same manner as described above, using the appropriate Fmoc-protected amino acid of formula (II) to yield resin-bound compound of Formula (III).

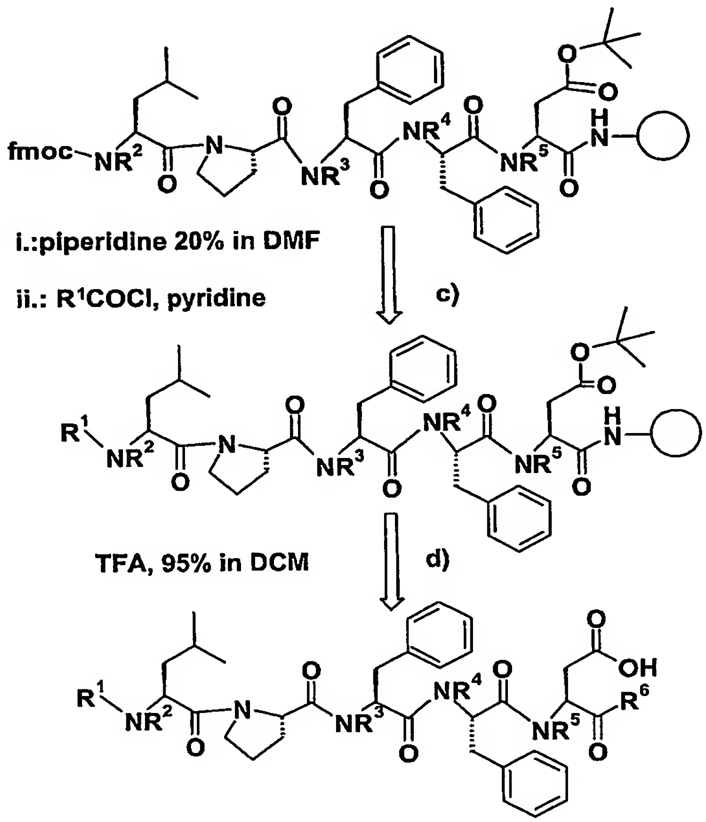
After the fifth Fmoc-amino acid-coupling step, the group R^1 is introduced as follows.

c) Introduction of R^1:

The Fmoc moiety is removed from compound of Formula (III) as described above in the deprotection step and the group R^1 is introduced.

For compounds of formula I in which R^1 is an unsubstituted acyl group (scheme 2), a freshly prepared solution of R^1COCl in pyridine (20 equivalents) is shaken with the resin-bound compound (compound of formula III) for two to 20 hours at 25 to 40°C (scheme 2). The resin is extensively washed with DCM, DMF, THF and again DCM three times before being submitted to the cleavage step. The resulting compound wherein the R^1 moiety is present is described by Formula (IV).

Scheme 2:



(III)

(IV)

(I)

d) Cleavage step:

A freshly prepared solution of 95% TFA in DCM is shaken with the resin for one to four hours at room temperature. This cleaves the compound from the resin as a C-terminal amide and removes the TFA-sensitive protecting groups. The solution is collected and the treatment is repeated two times. The solutions are collected and evaporated to dryness. The crude compound of Formula (I) is purified by preparative HPLC using conditions as described below.

Tritiation protocol :

Tritiated derivatives of compound of Formula I can be prepared following the general protocol coupling a 3,4-dehydro-proline residue in step 4.

The Dehydro-Pro peptide (2 mg) is mixed with 10% palladium on calcium carbonate (10 mg) and DMF (1 ml) in a tritiation vessel. The mixture is stirred under tritium gas (5 Ci) for

3 hours. The solution is filtered and labile tritium removed by repeated evaporations to dryness with ethanol.

Crude reaction mixture was purified by high performance liquid chromatography in the following system: On a Vydac C18 Protein and Peptide column (250 x 9.6 mm) using a water: acetonitrile: TFA gradient system. The detection is performed by a radioactive detector and a UV detector (220 nm). Product collected, evaporated to dryness, re-dissolved in dispensing eluent.

The other purifications are performed as followed: Preparative HPLC Waters Prep LC 4000 System equipped with columns Prep Nova-Pak® HR C186 µm 60Å, 40x30mm (up to 100 mg) or 40x300 mm (up to 1g). All the purifications were performed with a gradient of MeCN/H_2O 0.09% TFA.

The following building blocks are commercially available from Bachem, Switzerland, Fmoc-L-phenylalanine, Fmoc-(β-OtBu)-L-aspartic acid, Fmoc-L-leucine, Fmoc-L-proline, Fmoc-N-Me-L-phenylalanine, Fmoc-N-Me-(OtBu)-L-aspartic acid, Fmoc-N-Me-L-leucine.

Coupling reagents are commercially available from Novabiochem, Switzerland.

Generally, the peptide derivatives according to the general Formula (I) can be synthesized using standard peptide synthesis techniques either in solution or on solid phase. In both approaches typical coupling reagents are used, which are known to the person skilled in the art.

It will be also appreciated that where typical or preferred experimental conditions (i.e. reaction temperatures, time, moles of reagents, solvents etc.) are given, other experimental conditions can also be used unless otherwise stated. Optimum reaction conditions may vary with the particular reactants or solvents used, but such conditions will be appreciated by the person skilled in the art.

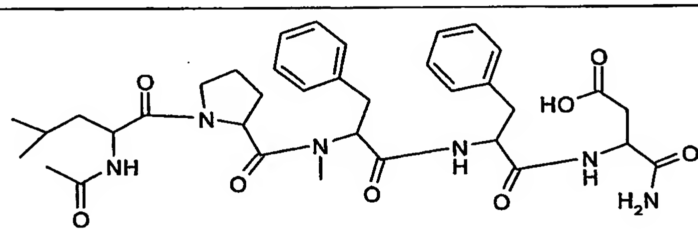
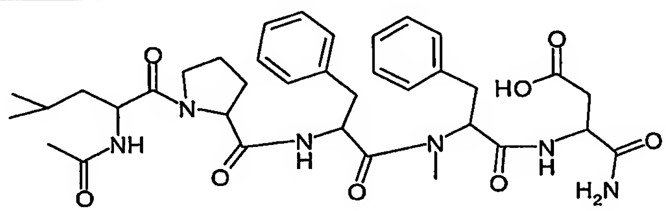
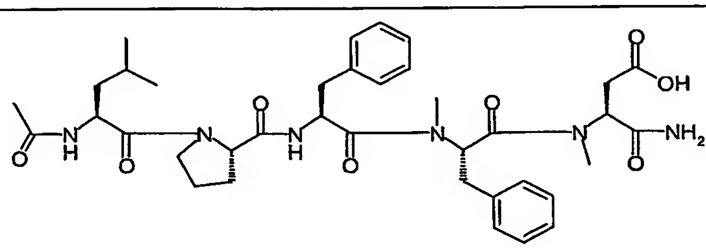
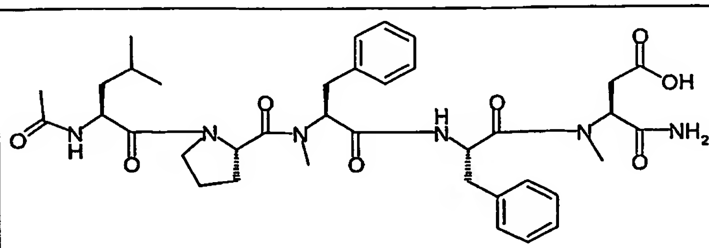
Examples

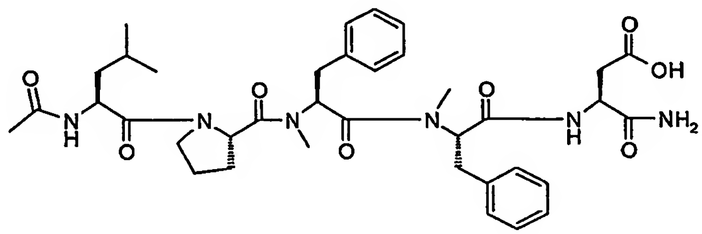
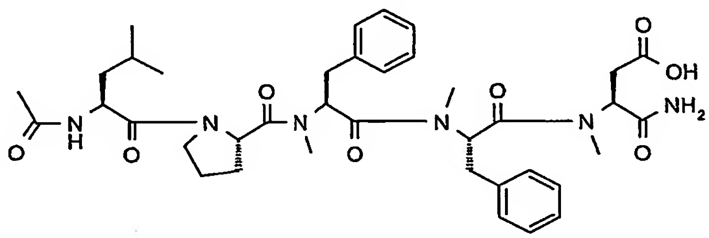
The compounds of Examples 1 to 7 are preferred embodiments of the invention. Structures of compounds of Examples 1 to 6 are presented in Table 1 below.

Compounds of Examples **1, 2, 3, 4, 5 and 6** may be synthesized according to protocol presented in scheme 1.

Compound of Example 7 stands for tritiated compound of Example 1 which may be synthesized according to protocol presented in scheme 1 using a 3,4-dehydro-proline amino acid instead of Proline in step 4. The peptide is then tritiated according to the tritiation protocol above. Compound of Example 7 had the specific activity of 42 Ci/mmol.

Table 1

Example	Structure
1	
2	
3	
4	

Example	Structure
5	
6	

The mass of compounds of Examples 1 to 7 are presented in Table 2 below.

Table 2

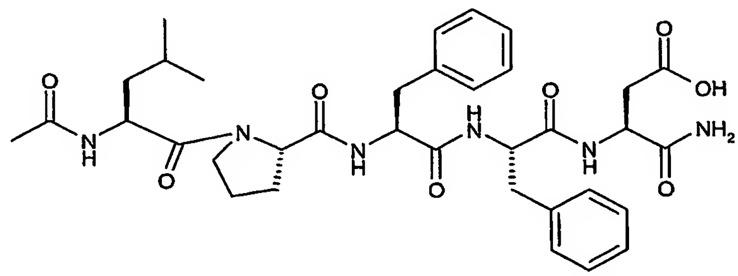
Example N°	Theoretical Mass	Experimental Mass
1	692.8	692.3
2	692.8	692.3
3	706.8	706.8
4	706.8	707.4

Example N°	Theoretical Mass	Experimental Mass
5	706.8	706.5
6	720.9	720.4
7	690.8	691.5

Comparative Example 8

The following compound is disclosed in WO 01/34631 (Axonyx Inc.), and is included as a reference compound.

<u>**Example 8:**</u>



<u>Example 9</u> : Biological assays

In vitro assays of peptide stability.

The stability of the compounds of the invention can be assayed in comparison with the reference compound **(Example 8)**.

Peptides were prepared as a 1μg/μl solution in water. 20 μl of the peptide solution was diluted in 80 μl of fresh human plasma or 10% rat brain homogenate (prepared in PBS). The resulting solution was incubated at 37°C for different times and the reaction was stopped by adding a complete cocktail of protease inhibitors. The bulk of plasma and brain proteins (but none of the peptide) were precipitated in cold methanol (mix/MeOH, 4/5, v/v) during one hour at -20°C. The precipitated proteins were pelleted by centrifugation (10000g, 10 min, 4°C). The supernatant, containing the peptide, was concentrated 5 times under vacuum and separated by reverse-phase HPLC. The area of the peak corresponding to the intact peptide was measured and compared with an equivalent sample incubated in PBS. The results of are listed in Table 3 as "t½ human plasma" and "t½ rat brain homogenate". The values compare favourably with those obtained for the reference compound of Example 8.

Table 3. *In vitro* half-lives of various peptides

Example n°	t½ human plasma	t½ rat brain homogenate
1	>24h	>24h
4	>24h	5h
5	>24h	>24h
6	>24h	>24h
8	>24h	15 min

In vitro assays of activity.

The activity of the compound of the invention in inhibiting the formation of aggregated fibrils can be tested by following the changes in fluorescence signal of a fluorophore that has an affinity for the amyloid fibrils.

Amyloid formation was quantitatively evaluated by the fluorescence emission of Thioflavine T (ThT) bound to amyloid fibrils, as reported by Levine[26] and also Soto *et al.*[27] Aliquots of Aβ1-42 (a synthetic peptide with the same sequence as the one deposited in amyloid plaques in Alzheimer's brain) at a concentration of 0.5 mg/ml prepared in 0.1M Tris, pH 7.4 were

incubated for 5 days at 37°C, gently swirled on a rotary shaker, in the absence or in the presence of different concentrations of the compounds. At the end of the incubation period, 50 mM glycine, pH 9.2 and 2 μM ThT were added in a final volume of 2 ml. Fluorescence was measured at excitation 435 nm and emission 485 nm in a Perkin Elmer, model LS50B fluorescence spectrometer. Using the analytical method[28], the percentage of inhibition of fibril formation caused by compounds of the invention can be calculated. The results are listed in Table 4 and shown graphically in Figure 1. Values correspond to the percentage of inhibition of amyloid formation and are expressed relative to the activity of the compound of Example 8 (100%).

Table 4. Inhibition of $A\beta_{1-42}$ fibril formation by compounds of the invention

Example n°	% Inhibition (Compound of Example 8 as 100%)
1	88
4	80
5	80
6	85

Cellular assay of activity.

Amyloid fibrils are cytotoxic, inducing cell death by apoptosis.[18] The ability of the compounds of the invention in preventing the amyloid formation can be evaluated by measuring the inhibition of the cytotoxicity in a cell assay.

Aliquots of $A\beta_{1-42}$ at a concentration of 0.5 mg/ml prepared in 0.1M Tris, pH 7.4 were incubated alone or in the presence of different concentrations of the compounds for 36h at 37°C, gently swirled on a rotary shaker. At the end of the incubation period, an aliquot of the solution was added to the medium of PC12 cells to reach a final concentration of Aβ of 5.5 μM. The cells were incubated for 24h and thereafter the cellular viability was evaluated using the MTT kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The results, listed in Table 5, are reported as the percentage of inhibition of

amyloid cytotoxicity, versus Aβ incubated alone and expressed relative to the activity of the compound of Example 8 (100%).

Table 5. Inhibition of amyloid cytotoxicity for compounds of the invention	
Example n°	% inhibition of cell death (Compound of Example 8 as 100%)
1	150
6	80

Blood-brain barrier permeability studies.

The ability of compound of the invention to cross the BBB can be checked by capillary depletion experiments and the kinetics of their penetration into the brain can be measured.

a) Capillary depletion and blood washout

Capillary depletion is used to assess the penetration into the brain of compounds of the invention. A "wash-out" step removes blood from the brain so that levels of the compounds of the invention present in the brain parenchyma can be measured.

Capillary depletion studies[27] were done in male CD-1 mice (28-36g). Mice are anaesthetized with i.p. urethane (40%) and the left jugular vein is exposed. A tritium labelled peptide of example 15 is injected i.v. . Before sacrificing the animals, blood is collected from the carotid artery (group 1) or from the descending aorta (group 2). After collection of blood, mice of group 1 are sacrificed or blood is removed by injecting 20 ml lactated Ringer's solution (7.19 g/l NaCl, 0.3 g/l KCL, 0.28 CaC12, 2.1 g/l $NaHCO_3$, 0.16+ g/l KH_2PO_4, 0.37 g/l $MgCl_2 \cdot 6H_2O$, 0.99 g/l D-glucose, 10 g/l bovine serum albumin, pH 7.4) into the left ventricle of the heart for 30 sec, which removes more than 95% of the vascular contents of the brain (blood brain washout, group 2).

The brain/serum ratio (µl/g) is evaluated by the equation: Brain/serum ratio = (cpm/g brain)/(cpm/µl serum). The cerebral cortex is weighed and homogenized in a physiological

buffer (10 mM HEPES, 140 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Na H_2PO_4, and 10 mM D-glucose, pH 7.4). Dextran solution (1.6 ml of a 26% solution) is then added to the homogenate. After centrifugation (5,400 g, 15 min, 4°C), brain vasculature (pellet) and parenchyma (supernatant) are separated and the radioactivity determined in each fraction.

After 10 min post i.v injection of radioactive derivative of Example 1 (compound of Example 7), more than 70% of the radioactivity is recovered in the parenchyma. The percentage of radioactivity associated with the capillary fraction with or without blood wash out is 24 % and 29%, respectively. These data show that the majority of compound 1 crosses the BBB. In addition, it proves that the peptide of Example 1 is weakly binding to the luminal surface of the capillaries and is not sequestrated into the endothelial cells.

b) Blood brain barrier permeability study:

The kinetics of penetration of compound of the invention into the brain can be evaluated through blood brain barrier permeability experiments. The percentage of injected peptide found in the brain can then be calculated.

Mice are anaesthetized with i.p. urethane (40%) and the left jugular vein is exposed. 0.2 ml of lactate Ringer's solution (7.19 g/l NaCl, 0.3 g/l KCL, 0.28 CaCl2, 2.1 g/l $NaHCO_3$, 0.16+ g/l KH_2PO_4, 0.37 g/l $MgCl_2 \cdot 6H_2O$, 0.99 g/l D-glucose, 10 g/l bovine serum albumin, pH 7.4) containing 1% BSA and tritium labelled peptide ("hot") of Example 7 (100 000 cpm/ml of tritium radioactive peptide) is injected. Arterial blood is collected from the right carotid artery at different time points following the labelled peptide injection. Serum is obtained by centrifugation (4800 g, 10 min, 4°C). Following arterial blood collection, the mice are decapitated and the whole brains, except the pineals and pituitaries, are harvested and weighed. The amounts of radioactivity in brain and serum are determined after an overnight solubilization step in TS-2 solution (RPI, Mount Prospect, IL) at 40°C. The brain serum ratio of total radioactivity was determined over time from 1 min up to 120 min after injection. The brain to serum ratio (µl/g) is estimated by the equation: Brain/serum ratio= (cpm/g brain)/(cpm/µl serum).

The representation of the brain to serum ratio versus time allows the derivation of the influx rate, Ki (slope) and the volume of distribution (Y intercept), Vi. The influx rate (Ki, microl (serum)/g (tissue weight)-min) represents the rate at which compounds move from the circulation to the brain. The volume of distribution (Vi, microl (serum)/g (tissue weight)) is the apparent volume of material which is distributed to the brain parenchyma at time 0 min. Results are presented in Table 6.

Table 6. Brain uptake study of compound of Example 7

Injection (i.v.) composition	K_i (microl/g-min)	V_i (microl/g)
Hot*	3.54 +/- 0.29	50.6 +/- 12.4

<u>*Hot: Radioactively labeled compound</u>

Ki: influx rate

Vi: volume of distribution

The influx rate (Ki), with radioactive peptide, alone was 3.54 +/- 0.29 microl/g-min with a Y intercept of about 50 microl/g (Table 4). This indicates that compound of Example 1 penetrates the brain.

The percentage of injected dose of compound of example 7, taken up per g of brain, was calculated (see Figure 2). The maximum value was 0.464% and half of this value was reached 1.4 min after injection. This data suggests a rapid brain uptake of compound of Example 7.

HPLC analysis of brain samples showed that 12% of intact peptide was recovered 20 min post i.v. injection.

More than 0.06% of the injected compound was recovered intact in the brain within the 20 min post injection.

***In vivo* studies using an animal model of cerebral Aβ deposition.**

The inhibitory activity the compound of the invention in the formation of amyloid deposits by can be visualized *in vivo* by staining cerebral tissue sections with anti-$A\beta_{1-42}$ antibodies in the presence and absence of a peptide of the invention.

Male Fischer-344 rats weighed 250-300g and were 3-4 months of age at the time of arrival. The animals were housed 2 per cage, maintained on a 12 h light-dark cycle with access to food and water *ad libitum* and were habituated to their new environment for 2-3 weeks prior to surgery. Surgery was performed under sodium pentobarbital (50 mg/kg, i.p.) anaesthesia. Atropine sulphate (0.4 mg/kg) and ampicillin sodium salt (50 mg/kg) were injected sub-cutaneously once the animals were anaesthetized. $A\beta_{1-42}$ was dissolved in dimethylsulfoxide (DMSO) and then diluted with water to a concentration of 16.7% DMSO. The animal received a bilateral injection of 5.0 nmol $A\beta_{1-42}$ into each amygdale by using a Kopf stereotaxic instrument with the incisor bar set at 3.3 mm below the interaural line. Injection coordinates measured from the bregma and the surface of the skull (AP -3.0, ML ± 4.6, DV -8.8) were empirically determined based on the atlas of Paxinos and Watson. A volume of 3.0 µl of the solution of $A\beta_{1-42}$ at 5.0 nmol was administered over 6 min (flow rate 0.5 µl/min) using a CMA/100 micro syringe pump. The cannula was left *in situ* for 2 min following injection, then it was withdrawn 0.2 mm and left for 3 min, and following these 5 min the cannula was slowly withdrawn. Following surgery the animals were placed on a heating pad until they regained their righting reflex. The animals were treated with compound of Example 1 and of Example 8. The peptides, solubilized in a 0.9% NaCl at the concentration of 4.4 mM were injected s.c (0.5 ml per injection), 4 times a week during 7 and a half weeks. After treatment with the compounds, animals were sacrificed by an overdose of sodium pentobarbital (150 mg/kg, i.p.), perfused transaortically. For histology, serial coronal sections (40 µm) of the brain were cut, placed in ethylene glycol cryoprotectant and stored at –20°C until stained. Tissue sections were stained with anti-$A\beta_{1-42}$ antibodies, as described. An image analysis system was used to determine the size of the amyloid deposits. These data were analysed by a two-way ANOVA followed by a Newman-Keuls' multiple range test for *post hoc* comparisons.

The photographic results for the compound of Example 1 in comparison with vehicle and the compound of comparative Example 8 are shown in Figure 3A. Graphical results depicting the amyloid area are shown in Figure 3B. It is clear that the compound of Example 1 substantially reduces the amyloid area.

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